Harpullia pendula Planch leaves: phenolics, *in vitro* antioxidant and α-amylase inhibitory activity

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Background and objective
Harpullia pendula Planch leaves belong to the Sapindaceae family. The study aimed to investigate the phenolic constituents and evaluate the antioxidant and α-amylase inhibitory activities of the plant's extracts and its major compounds.
Materials and methods
The compounds were isolated through chromatographic techniques from the defatted ethanolic extract (DAEE). Their structures were determined by ultraviolet, mass spectrometer, and nuclear magnetic resonance spectroscopy.
Results
The flavonoids kaempferol 3-*O*-(6″galloyl)- apiofuranosyl (1‴→2″)-β-galactopyranoside, kaempferol-3-*O*-β-glucopyranosyl(1‴→6″)-β-glucopyranoside, kaempferol 3-*O*-(6″galloyl)-apiofuranosyl (1‴→2″)-β-galactopyranoside, rutin, vitexin, isovitexin, orientin, quercetin, kaempferol; the tannins ellagic acid, gallic acid, methyl gallate, 2,6-di-*O*-galloyl(α/β)glucoside, 2,3-di-*O*-galloyl(α/β)glucoside, and tetragalloyl glucoside in addition to two benzene acetic acid derivatives, harpulliaside A and cavaol B, were isolated from the total bioactive ethanolic extract (TEE). The TEE and the DAEE of *H. pendula* have a total phenolic content of 255.5± 7.18 and 222.9±6.43 mg gallic acid equivalents/g extract, respectively, and a total flavonoid content of 111.6±3.2 and 102.6±2.6 mg quercetin equivalents/g extract, respectively. With respect to the *in vitro* study, DAEE, TEE, and methyl gallate showed an interesting inhibitory activity on 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) [half maximal inhibitory concentration (IC_{50}): 13.3±0.4, 17.7±0.7, and 19.4±0.08 μg/ml, respectively], nitric oxide (IC_{50}: 12.8±2.54, 18.3±1.6, and 29.8± 1.00 μg/ml, respectively), and α-amylase (IC_{50}: 6.1±0.554, 14.4±0.681, and17.5± 0.003 μg/ml, respectively).
Conclusion
H. pendula extracts are rich in phenolic compounds; the aforementioned results suggest that DAEE, TEE, and methyl gallate may be potentially useful in hegemony of obesity and diabetes.

Keywords:
Antioxidants, α-amylase, cavaol B, flavonoids, gallic derivatives, *Harpullia pendula*, harpulliaside A

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Introduction

Sapindaceae (Soapberry family) is a family of flowering plants comprising 37 species; members of this family have been widely studied for their antioxidant, anti-inflammatory, insecticidal, and antidiabetic properties [1,2]. Plants belonging to this family generally contain flavonoids, phenolic acids, saponins, and tannins in their leaves [3]. *Harpullia* is a genus of this family [4]; phytochemically, it is characterized by triterpenoid saponin [5]. They were reported for several biological activities such as being antifungal, cytotoxic, antiviral, antibacterial, molluscicidal, miracidicidal, and cercaricidal [6]. *Harpullia pendula* Planch is a small to medium-sized plant that is 6–9 m in height. It is also known as tulipwood or tulip lancewood and is mainly planted for ornamental purposes; they grow naturally from India eastwards to the Pacific Islands [7]. Bark, fruits, and seeds of *H. pendula* are used by ethnic communities as leech repellent, hair wash, and for antirheumatic purposes. The fruit extract mixed with coconut oil and castor oil is used for digestive problems and also as an appetizer [8]. Acylated triterpenoid saponins were isolated from the seeds and their anti-inflammatory effect was reported [9]. *H. pendula* exhibited radical scavenging and antioxidant potential [10]. Hyperglycemia induces generation of reactive oxygen species in both types of diabetes, and the onset of diabetes is closely associated with oxidative stress and development of complications. This encourages the

authors to determine the hypoglycemic effect in correlation to the scavenging ability of the plant extracts. Herein, we report the isolation and structural elucidation of phenolic compounds from the leaves of *H. pendula* and their potential as oxidation and α-amylase inhibitors.

Materials and methods

General experiment

The compounds' structures were determined through spectroscopic methods using the ultraviolet (UV) and visible absorption spectrometer (UV-VIS; Labomed Inc., Los Angeles, CA, USA) to detect the UV absorbance of the isolated compounds, in the range of 200–500 nm in methanol and with shift reagents. Nuclear magnetic resonance (NMR) spectrophotometer (Jeol, Tokyo, Japan; 500 MHz for determination of ^{1}H NMR and 125 MHz for determination of ^{13}C NMR, solvents were used as internal standard) and electrospray ionization (ion trap) mass spectrometer (ESI-MS; Thermo Finnigan, San Jose, CA, USA) were used for the determination of molecular weight of the compounds. Column chromatography was carried out on Diaion HP-20 (Diaion Supelco-13606; Sigma-Aldrich, Bellefonte, Pennsylvania, USA), polyamide 6S (Riedel-De-Haen AG, Seelze Haen AG, Seelze, Germany), and Sephadex LH-20 (Pharmazia, Sweden). Paper chromatography (descending) was carried out with Whatman No. 1 and 3 mm papers, using solvent systems: 6% HOAc (H_2O–HOAc, 94 : 6) and BAW (*n*-BuOH–HOAc–H_2O, 4 : 1 : 5) and upper layer: 15% HOAc (H_2O–HOAc, 85 : 15). The aglycone and sugar moieties were identified by complete acid hydrolysis for *O*-glycosides followed by comparative co-chromatography with authentic specimens (Sigma-Aldrich). Solvents used for plant extraction were purchased from SDFCL (Mumbai, India). Materials used for determination of total phenolic (TP) and total flavonoid (TF) compounds were as follows: Folin–Ciocalteu reagent, gallic acid, quercetin, $NaNO_2$, Na_2CO_3, $AlCl_3$ (Merck, Germany). Materials used for the determination of antioxidants were as follows: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), $K_2S_2O_8$, sodium nitroprusside, Griess reagent (1% sulfanilamide in 5% *ortho*-H_3PO_4 and 0.1% naphthyl ethylenediamine dihydrochloride), phosphate buffered α-amylases (EC 3.2.1.1), and 3,5-dinitrosalicylic acid (DNS); all chemicals were from Sigma-Aldrich.

Plant material

Fresh leaves of *H. Pendula* Planch were collected in March from the Orman Garden (Giza, Egypt). Voucher specimens (no. H-5-7) were kept in the garden herbarium.

Preparation of plant extracts

Extraction

Dried powdered leaves of *H. Pendula* (700 g) were extracted with 70% aqueous ethanol, and the solvents were evaporated to dryness to give 197 g of ethanolic extract (TEE); 160 g of this extract was suspended in water and defatted with dichloromethane to give 140 g of defatted ethanolic extract (DAEE).

Isolation

The DAEE extract was fractionated on Diaion HP-20, eluted with 100% water and thereafter the polarity was decreased by adding methanol 25, 50, 70, and finally 100% to obtain five substantial fractions (fractions A–E). Fraction A was subjected to preparative paper chromatography (Whatman No. 3 mm descending) using 6% acetic acid in water, purified on Sephadex LH-20 column using MeOH/H_2O, and crystallized from methanol to give gallic acid, methyl gallate (MG), 2,6-di-*O*-galloyl(*α*/*β*) glucoside, 2,3-di-*O*-galloyl(*α*/*β*)glucoside, tetragalloyl glucoside, and ellagic acid. Fraction B was applied to preparative paper chromatography eluted with 15% acetic acid in water and purified as fraction A to give, kaempferol-3-*O*-β-glucopyranosyl(1‴→6″)-β-glucopyranoside kaempferol-3-*O*-(6″galloyl) - apiofuranosyl (l‴→2″)-β-galactopyranoside, rutin. Fraction C was applied on polyamide column eluted with H_2O/EtOH gradient to offer two benzene acetic acid derivatives – harpulliaside A and cavaol B – in addition to vitexin, isovitexin, and orientin. Fraction D offered quercetin and kaempferol upon application on column Sephadex LH-20 and elution with 50% MeOH/H_2O.

Spectral characterization of compounds

Kaempferol-3-O-β-glucopyranosyl (1‴→6″)-β-glucopyranoside (kaempferol-3-O-gentiobioside): (1) Paper chromatography: R_f 0.34 (BAW), 0.49 (15% HOAc); UV dark purple, UV/NH_4 dark greenish yellow. UV λ_{max} (nm): MeOH: 266, 296sh, 348; +NaOMe: 275, 326, 400; +$AlCl_3$: 275, 305, 354, 396; +$AlCl_3$/HCl: 276, 303, 346, 396; +NaOAc: 275, 313, 389; +NaOAc/H_3BO_3: 267, 297sh, 352. ^{1}H NMR (500 MHz, DMSO-d_6): δ 13.14 (1H, s, 5-OH), 8.07 (2H, d, *J*=9.2 Hz, H-2′,6′), 6.92 (2H, d, *J*=9.2 Hz, H-3′,5′), 6.45 (1H, d, *J*=2.1 Hz, H-8), 6.21 (1H, d, *J*=2.1 Hz, H-6), 5.22 (1H, d, *J*=7.3 Hz, glucosyl anomeric H1″), 4.50 (1H, d, *J*=7.9 Hz, glucosyl anomeric H1‴). ^{13}C NMR (125 MHz,

DMSO-d_6): δ kaempferol: 156.6 (C-2), 133.1 (C-3), 177.5 (C-4), 161.6 (C-5), 99.8 (C-6), 165.8 (C-7), 94.6 (C-8), 158.0 (C-9), 104.2 (C-10), 120.8 (C-1′), 132.0 (C-2′,6′), 116.1 (C-3′,5′), 161.6 (C-4′); δ glucose: 105.2 and 104.2 (C-1″, C-1‴), 73.90 and 73.20 (C-2″,C-2‴),78.4 (C-3″,C-3‴), 71.3 and 71.1 (C-4″,C-4‴), 78.1 and 77.7 (C-5″,C-5‴), 69.8 and 62.5 (C-6″,C-6‴). ESI-MS (*m/z*): 611 $[M+H]^+$.

Kaempferol-3-O-(6″galloyl)-apiofuranosyl (l‴→2″)-β-galactopyranoside: (2) Yellow powder. UV λ_{max} (nm): MeOH: 267, 352; NaOMe: 275, 323, 392; $AlCl_3$: 275, 303sh, 347, 392; $AlCl_3$+HCl: 274, 303sh, 345, 392; NaOAc: 274, 304sh, 382; NaOAc +H_3BO_3: 267, 309sh, 348. ESI-MS (*m/z*): 733 $[M+H]^+$, 581 $[M+H-152]^+$, 449 $[M+H-132]^+$, 287 $[M+H-294]^+$. ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.07 (2H, d, *J*=8.9 Hz, H-2′ and H-6′), 7.08 (2H, s, H-2″, 6″), 6.92 (2H, d, *J*=8.9 Hz, H-3′ and H-5′), 6.45 (1H, d, *J*=1.8 Hz, H-8), 6.21 (1H, d, *J*=1.8 Hz, H-6), 5.43 (1H, d, *J*=7.5 Hz, H-1‴), 5.17 (1H, d, *J*=l.4 Hz, H-1⁗), 3.00–3.90 (unresolved sugar protons). ^{13}C NMR (125 MHz, DMSO-d_6): δ 92.8 (C-8), 97.5 (C-6), 103.2 (C-10), 115.6 (C-3′ and C-5′), 120.7 (C-1′), 131.4 (C-2′ and C-6′), 132.9 (C-3), 155.1 (C-9), 156.2 (C-2), 159.8 (C-4′), 161.8 (C-5), 166.1 m(C-7), 175.9 (C-4); Sugar: Glucose 63.9 (C-6″),73.9 (C-5″) 70.9 (C-4″), 73.4 (C-3″), 74.6 (C-2″), 98.2 (C-1″). Apiose: 68.7 (C-5‴), 75.7 (C-4‴), 78.0 (C-3‴), 76. 6 (C-2‴), 108.8 (C-1‴). Galloyl: 109.1, 109.2 (C-2⁗, 6⁗), 119.8 (C-1⁗), 139.0 (C-4⁗), 145.9, 146.0 (C-3⁗, 5⁗), 166.3 (C-97⁗).

2,6-Di-O-galloyl(α/β)glucoside: (3) ^{1}H NMR (δ ppm D_2O): α-glucose: 5.75 (dd, *J*=8.1, 9.0 Hz, H-2-α), 5.46 (d, *J*=8 Hz, H-1-α), 4.93 (dd, *J*=5.1, 12.0 Hz), 4.87 (dd, *J*=2.1, 12.0 Hz, 2H-6), 3.6–3.86 (m, H-3, H-4, H-5); β-glucose: 5.42 (dd, *J*= 8.1, 9.0 Hz, H-2-β), 4.99 (d, *J*=8 Hz, H-1-β), 4.52 (dd, *J*= 5.1, 12.0 Hz), 4.63 (dd, *J*=2.1, 12.0 Hz, 2H-6). ^{13}C NMR (δ ppm): 90.08 (C-1), 75.62 (C-2), 73.39 (C-3), 69.68 (C-4), 74.00 (C-5), 63.44 (C-6); 95.01 (C-1), 72.56 (C-2), 73.39 (C-3), 68.80 (C-4), 75.63 (C-5), 63.24 (C-6); Galloyl moieties in α and β anomers: ^{1}H NMR (δ ppm): 7.11, 7.01, 7.00, 6.8 each (2H,s,H-2,H-6); ^{13}C NMR (δ ppm): 120.45, 120.31 (C-1), 109.23, 109.13 (C-2 and C-6), 145.43, 145.35, 145.30, 145.29 (C-3 and C-5), 138.69, 138.47 (C-4), 166.63, 166.49, 165.97, 165.94 (C=O).

2,3-Di-O-galloy(α/β)glucoside: (4) ^{1}H NMR (δ ppm D_2O): α-glucose: 5.42 (d, *J*=3.2 Hz, H-1-α), 4.91 (dd, *J*=8, 3.2 Hz H-2-α), 5.76 (t, H-3-α), 3.6–4 (m) (H-4, H-5, H-6, H-6′); β-glucose: 4.96 (d, *J*=7.5 Hz, H-1β), 5.06 (t, *J*=7.5 Hz, H-2β), 5.40 (t, H-3-β), 3.6–4.0 (m) (H-4, H-5, H-6, H-6′); ^{1}H NMR galloyl moieties in α and β anomers: 7.05 s, 7.06 s, 7.1 (2H,s,H-2,H-6). ^{13}C NMR (δ ppm): α-glucose: 89.2 (C-1), 75.2 (C-2), 72.2 (C-3), 68.2 (C-4), 72.2 (C-5), 60.6 (C-6); β-glucose: 94.4 (C-1), 73.1 (C-2), 75.4 (C-3), 68.2 (C-4), 76.6 (C-5), 60.6 (C-6). Galloyl moieties: ^{13}C NMR (δ ppm) 119.42, 119.15, 119.50 (C-1), 109.58, 109.06 (C-2 and C-6), 145.35, 145.05 (C-3 and C-5), 138.95, 138.65 (C-4), 164.81, 165.94, 166.23, 165.5 (C=O).

Harpulliaside A: (5) The compound was obtained from water as white amorphous powder and as yellow resin from methanol. UV λ_{max} (nm): MeOH: 266, 325. Negative ESI-MS (*m/z*): 533 $[M-H]^-$, 371 $[M-H-162]^-$. ^{1}H NMR (500 MHz, DMSO-d_6): δ: benzen ring: 6.98 and 6.95 (d, *J*=3 Hz, H-2, H-6); prenyl group: 3.6 (2H, m, H-7), 5.53 (t, 1H, H-8), 1.64 and 1.66 each (s, 2CH_3, H-10, H-11); lavandulyl group: 3.17 and 2.58 (dd, 2H, H-12), 2.46 (m, 1H, H-13), 1.60 (s, 3H, H15) 4.74 s, 4.44 (d, *J*=3.4, 2H, H-16), 2.04 (m, 2H, H-17), 0.5.21 (t, 1H, H-18), 1.50 and 1.56 (s, 2CH_3, H-20, H-21), 3.08–3.64 (H-2-H6); glucose moiety: 4.54 (d, 8.6, 1H, H-1′); acetic acid moiety: 5.04 (s, 1H, H22). ^{13}C NMR (125 MHz, DMSO-d_6): δ: benzen ring: 136 (C-1), 126.9 (C-2), 134.7 (C-3), 152.7 (C-4), 134.5 (C-5), 126.0 (C-6); prenyl group: 28 (C-7), 124.2 (C-8), 131.6 (C-9), 18.4 (C-10, CH_3), 26.2 (C-11, CH_3); lavandulyl group: 34.1 (C-12), 49.1 (C-13), 147.7 (C-14), 19.6 (C-15, CH_3), 111.8 (C-16), 34.1 (C-17), 123.7 (C-18), 131.4 (C-19, C), 18.2 (C-20, CH_3), 26.1 (C-21, CH_3); acetic acid moiety: 73.2 (C-22), 175.4 (C-23, C=O); glucose moiety: 105.2 (C-1), 74.6 (C-4, CH), 77.4 (C-3), 70.6 (C-4), 77.04 (C-5), 61.8 (C-6).

Cavaol B(6) ESIMS (*m/z*) 549 $[M-H]^-$, 387 $[M-H-162]^-$, NMR analysis showed signals similar to that of compound 5 except for additional signals at δ 3.44,3.36 and 63.3 ppm corresponding to CH_2 group and absence of one singlet of CH_3 group.

High-performance liquid chromatography quantification of major components

The two abundant compounds MG and gallic acid were quantified in the defatted extract according to the method reported by El Souda *et al.* [11].

Determination of total phenolic and flavonoid contents in the plant extracts

TP and TF and flavonoid assays were conducted using the modified method of Marinova *et al.* [12], wherein 0.5 g of powdered leaves was extracted using 50 ml of

80% aqueous methanol in an ultrasonic bath for 20 min. Then the mixture was centrifuged for 5 min at 14000 rpm. The supernatant was collected and used for TP and TF quantification.

Estimation of total phenolic content

TP was assayed using the Folin–Ciocalteu assay [12]. A volume of 1 ml of the extract or standard solution of gallic acid (20, 40, 60, 80, 100 mg/l) was used; 1 ml of Folin–Ciocalteu's phenol reagent was added to it, and the mixture was mixed and shaken. After 5 min, 10 ml of 7% sodium carbonate was added; the mixture was then completed to 25 ml with distilled water, mixed, and allowed to stand at room temperature for 90 min. The absorbance against the prepared reagent blank was determined at 750 nm using a spectrophotometer (Jasco V630 spectrophotometer, Guangdong, China (Mainland)). The TP content was expressed as mg gallic acid equivalents/g extract.

Determination of total flavonoid content

TF was determined using the $AlCl_3$ colorimetric method. A volume of 1 ml of plant extract or standard solution of quercetin (20, 40, 60, 80, 100 mg/l) was added to 4 ml of distilled water in a 10 ml volumetric flask. A volume of 0.3 ml of 5% $NaNO_2$ was added after 5 min, and 0.3 ml of 10% $AlCl_3$ was added and left for 6 min. Thereafter, 2 ml of 1 mol/l NaOH was added. The mixture was diluted to 10 ml with distilled water. The absorbance of the solution was measured at 510 nm. The results were expressed as mg quercetin equivalents (Qu)/g, and all samples were analyzed in triplicate.

Biological study

Determination of the antioxidant activity

ABTS radical scavenging assay: $ABTS^+$ assay was performed as described by Almeida *et al.* [13]. ABTS is a protonated radical and has a maximum UV absorbance at 734 nm, which decreased with the scavenging of the proton radicals [14]. The reagent was prepared by addition of 5.0 ml of 7 mmol/l ABTS to 88.0 µl of 140 mmol/l of $K_2S_2O_8$ solution and allowed to stand in dark for 16 h; after incubation, the ABTS reagent was diluted by adding 95% ethanol so that an absorbance of 0.7±0.05 was achieved. A volume of 10.0 µl of different sample concentrations (100, 250, 500, 1000 µg/ml test) or distilled water (control) was mixed with 1.0 ml of ABTS reagent. Absorbance was taken against the blank at 734 nm after incubation for 6 min, and ABTS radical scavenging activity was calculated according to the following equation: SA%= $absorbance_{control}$–$absorbance_{sample)}$/$absorbance_{control}$ ×100.

Nitric oxide (NO) radical scavenging activity: NO scavenging activity of tested compounds was determined by using a sodium nitroprusside generating NO system. The generated $NO^{\cdot}$ in aqueous solution at physiological pH reacts with oxygen to produce nitrite ions, which were measured by the Griess reagent [15] which constitute 1% sulfanilamide in 5% *ortho*-H_3PO_4 and 0.1% naphthyl ethylenediamine dihydrochloride. The reaction mixture (2 ml) containing various concentrations of the extracts, standard compounds (100, 250, 500, 1000 µg/ml), and sodium nitroprusside (final concentration, 10 mmol/l) in a PBS of pH 7.4 was incubated at 25°C for 150 min. After incubation, 1 ml samples of reaction mixtures were removed and diluted with 1 ml Griess reagent. The absorbance of these solutions was measured at 540 nm against the corresponding blank solution.

%Scavenging=(absorbance of control–absorbance of test sample/absorbance of control]×100.

Determination of α-amylase inhibitory activity as a carbohydrate hydrolyzing enzyme

α-Amylase (EC 3.2.1.1) catalyzes the hydrolysis of α-1,4 glycosidic linkages in starch and other related carbohydrates. In particular, α-amylase participates in glucose digestion and is considered as a key enzyme that can control postprandial hyperglycemia. The principle of the assay depends on the fact that reducing sugars have the property to reduce many reagents; chemically they form an aldehyde or ketone in basic solution, and the aldehyde group of glucose converts DNS to its reduced form (3-amino-5-nitrosalicylic acid); the formation of the latter results in a change in the amount of light absorbed at a wavelength of 540 nm. By using this method, the percentage of α-amylase inhibitory activity and IC_{50} values of each extract was calculated as follow:

$$\alpha\text{-Amylase activity (\% inhibition)} = (Abs_{Control} - Abs_{sample})/Abs_{Control} \times 100)$$

A volume of 0.3 ml of enzyme solution (3 U) was premixed with extracts at various concentrations (100, 250, 500, 1000 µg/ml); thereafter, 0.5 ml of starch was added to start the reaction. The reaction mixture was carried out at 37°C for 5 min and terminated by the addition of 2 ml of DNS reagent. The mixture was then heated for 15 min at 100°C and diluted with 10 ml of bidistilled water in an ice bath. The α-amylase activity was determined by measuring the absorbance at 540 nm [16].

Statistical analysis

The data of biochemical assessments are expressed as mean±SD and one way analysis of variance (ANOVA) followed by post-hoc tests (least significant difference) to compare the groups. The statistical analysis was carried out by using computer software Statistical Package for the Social Sciences (SPSS) version 16 (SPSS Inc. Released 2007, SPSS for Windows, and Version 16.0. Chicago, SPSS Inc.). Values were considered statistically significant ($P<0.05$).

Results

Structure elucidation of compounds

Chromatographic kaempferol-3-*O*-β-glucopyranosyl (1‴→6″)-β-glucopyranoside, kaempferol-3-*O*- (6″ galloyl)-apiofuranosyl (1‴→2″)-β-galactopyranoside rutin, vitexin, isovitexin, orientin, quercetin, kaempferol and tannins ellagic acid, gallic acid, methyl gallate, (α/β)2,6-di-*O*-galloylglucoside, (α/β) 2,3-di-*O*-galloyl glucoside and tetragalloyl glucoside in addition to two benzene acetic acid derivatives, harpulliaside A and cavaol B, were isolated. The compounds were specified by UV, ^{1}H, ^{13}C NMR, and ESI-MS, complete and mild acid hydrolysis, and comparison with those in the literature were identified as follows:

Kaempferol-3-*O*-β-glucopyranosyl(1‴→6″)-β-glucopyranoside (kaempferol-3-*O*-gentiobioside): (1) the spectral data were matching with that previously reported for kaempferol-3-*O*-β-glucopyranosyl(1‴→6″)-β-glucopyranoside [17].

Kaempferol-3-*O*-(6″galloyl)-apiofuranosyl(1‴→2″)-β-galactopyranoside: (2) the NMR data were in accordance with that reported for – kaempferol-3-apiofuranosyl(1‴→2″)-β-galactopyranoside [18] and for kaempferol-3-*O*-(6-β-*O*-galloyl-β-glucopyranoside) [19]. From these collective data the compound was identified as kaempferol-3-*O*-(6″galloyl)-apiofuranosyl (1‴→2″)-β-galactopyranoside.

2,6-Di-*O*-galloyl(α/β)glucoside: (3) the data coincide with that previously reported [20].

2,3-Di-*O*-galloy(α/β)glucoside: (4) the NMR data were identical to that previously reported [21].

Spectral data of compounds: vitexin, isovitexin, orientin, kaempferol, rutin, quercetin, gallic acid, methyl gallate (MG), and ellagic acid data were in good accordance with previously published data [22–25].

Harpulliaside A: (5) the negative ESI-MS and NMR spectral data are in accordance with that recently reported for harpulliaside A [26].

Cavaol B: (6) 4-*O*-β-glucopyranosyl-5-(2-(3-*O*-methyl-2-*O*-butenyl)-3-methylidene-n-butanol)-3-(3-methyl-2-butenyl)-22-hydroxy-22-phenylacetic acid.

The compound showed NMR spectral signals similar to that of harpulliaside A, with additional signals at δ 3.44, 3.36, and 63.3 ppm corresponding to the CH_2 group and only four methyl groups. The negative ESI-MS (*m/z*) 549 $[M-H]^-$, 387 $[M-H-162]^-$ represented the loss of a hexose moiety. Also, an additional 16 amu for the aglycon suggesting that C_{15} methyl group was replaced by CH_2OH. The data match with that of cavaol B [27]. The structures of the compounds 1-6 were illustrated in Fig. 1.

Total phenolic and total flavonoid contents

The phenolic and flavonoid contents of both TEE and DAEE are illustrated in Table 1. Their amounts were lower in the DAEE extract.

In-vitro assessment

With respect to the *in-vitro* study, both extracts exhibited inhibitory effect on ABTS, NO, and α-amylase in linear relationship with the concentration of inhibitors (concentration dependent) (Figs 2–4). The DAEE possessed higher scavenging ability than the TEE and MG (Tables 2 and 3). The DAEE shows more potent suppression power than the TEE on α-amylase (carbohydrate hydrolyzing enzyme) in comparison with standard inhibitor drugs acarbose and maltose. Meanwhile, MG showed more inhibitory effect than maltose.

Antioxidant activity

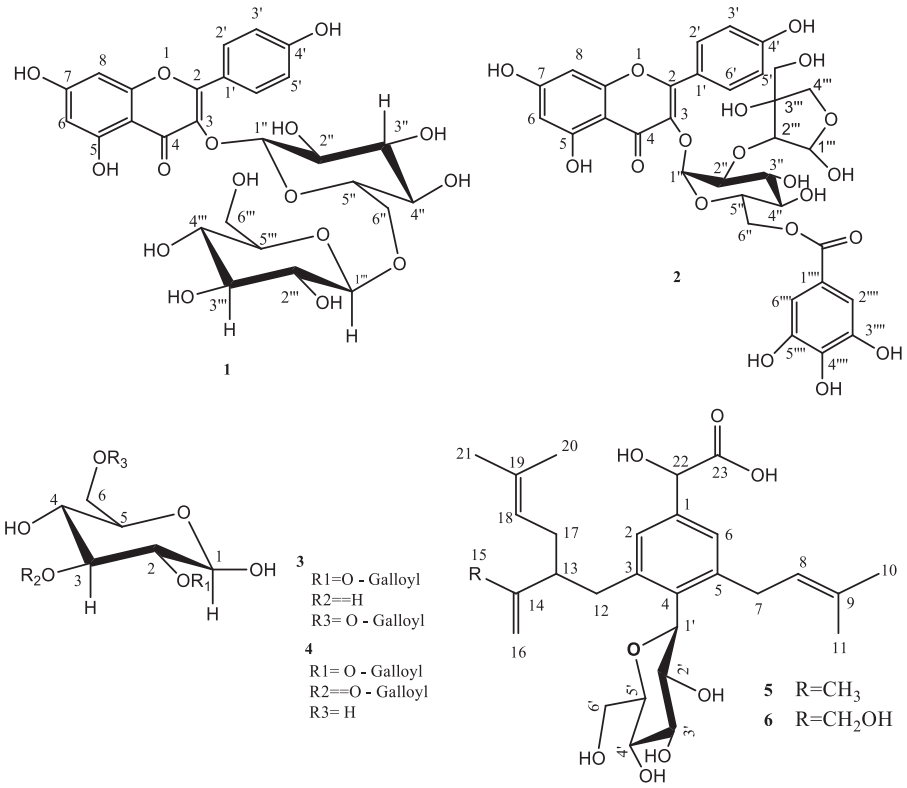
ABTS radical scavenging ability

The ABTS free radical scavenging effect of different extracts of *H. pendula* leaves TEE, DAEE, and major pure isolated compound MG was illustrated in Fig. 2 as percent inhibition. All the tested extracts show appreciable free radical scavenging activities, compared with vitamin C as a reference compound. DAEE has the strongest radical scavenging activity at different concentrations followed by TEE and MG. A dose–response relationship is found in the ABTS radical scavenging activity. The concentrations that produce 50% inhibition are compiled in Table 2.

Nitric oxide scavenging capacity

The NO free radical scavenging capacitiy of the different extracts of *H. Pendula* TEE, DAEE, MG, and vitamin C

Figure 1



Chemical structure of compounds 1-6.

Table 1 Total phenolic and total flavonoid content in the total and defatted ethanol extracts of *Harpullia pendula* leaves

Samples	Total phenolics (mg GAE/g extract)	Total flavonoids (mg Qu/g)
Total ethanol extract	255.5±7.18[a]	111.6±3.2[a]
Defatted extract	222.9±6.43[b]	102.6±2.6[b]

Data are represented by the mean±SE, *n*=3. GAE, gallic acid equivalent; Qu, quercetin equivalent. Mean values in the same column bearing the same superscript do not differ significantly ($P \leq 0.05$).

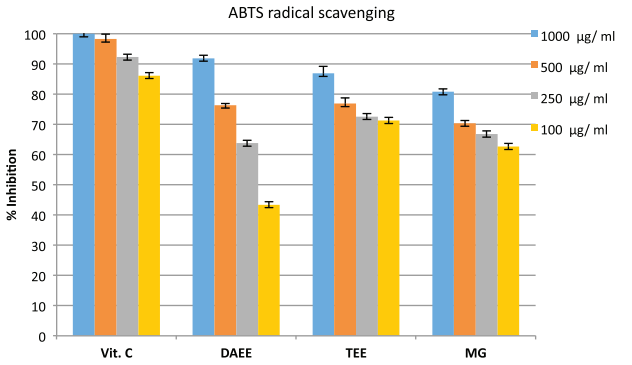
Table 2 Fifty percent scavenging concentration of *Harpullia pendula* tested substances in ABTS and nitric oxide assays

Extracts	IC_{50} (µg/ml)	
	ABTS	NO
TEE	17.7±0.70[*,d]	18.3±1.60[*,d]
DAEE	13.3±0.40[c]	12.8±2.54[c]
MG	19.4±0.08[b]	29.8±1.00[b]
Vitamin C	21.2±0.24[a]	32.5±1.06[a]

Values are presented as mean of triplicates±SD. Data were analyzed by one-way analysis of variance followed with post-hoc for multiple comparisons. ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; DAEE, defatted ethanolic extract; IC_{50}, half maximal inhibitory concentration; MG, methyl gallate; NO, nitric oxide. Groups bearing the same letter have no significant difference between them. *Significantly different from different extracts and standard value ($P<0.05$).

are represented in Fig. 3 as percent inhibition. The expression of antioxidant activity is thought to be associated with the development of reductions, as these

Figure 2



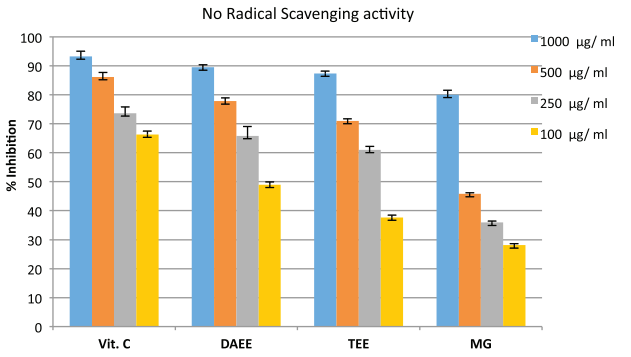
Free radical scavenging effect of *Harpullia pendula* extracts at different concentrations. All data are mean of triplicates. Data were analyzed by one-way analysis of variance and presented as mean ±SD ($P<0.05$).

species are known to be free radical chain terminators. The most reducing capacity is also considered for DAEE and has the strongest radical scavenging activity at different concentrations followed by TEE and MG. The concentrations that produce 50% inhibition are recorded in Table 2.

α-Amylase inhibitory effect

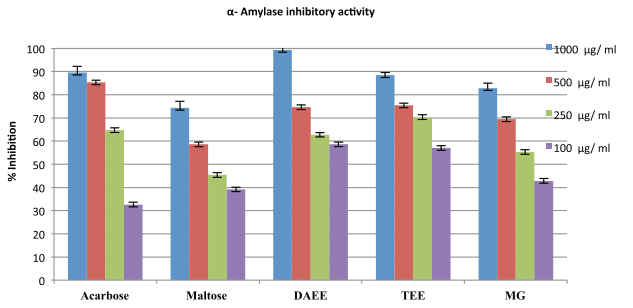
The reducing ability of AEE, DAEE and MG of *H. pendula* on α-amylase carbohydrate hydrolyzing enzyme activity compared with maltose and acarbose

Figure 3



Percent of nitric oxide inhibition effect of *Harpullia pendula* extracts at different concentrations. All values are mean of triplicates. Data were analyzed by one-way analysis of variance and recorded as mean±SD ($P<0.05$).

Figure 4



Percent of α-amylase inhibition of *Harpullia pendula* extracts at different concentrations. All values are mean of triplicates. Data were analyzed by one-way analysis of variance (ANOVA) and recorded as mean±SD ($P<0.05$).

(standard compounds) is demonstrated in Fig. 4. The inhibition of α-amylase by TEE, DAEE, and MG also appears to be dose dependent and is found to have the highest significant (at $P<0.05$) inhibiting activity 88.4±1.79, 99.3±1.55, and 82.8±1.15% at inhibitor concentration of 1000 µg/ml, respectively, compared with reference compounds acarbose and maltose, which reached 89.5±1.81 and 74.34±1.42% at inhibitor concentration of 1000 µg/ml, respectively. The concentrations that reproduce 50% inhibition are summarized in Table 3.

Table 3 Fifty percent inhibiting concentration of *Harpullia pendula* tested substances on α-amylase enzyme

Extracts	IC_{50} (µg/ml)
TEE	14.4±0.681^{*,e}
DAEE	6.1±0.554^{d}
Methyl gallate	17.5±0.003^{b}
Acarbose	15.7±0.630^{c}
Maltose	23.4±0.975^{a}

Values are presented as mean of triplicates±SD. Data were analyzed by one-way analysis of variance followed with post-hoc for multiple comparisons. DAEE, defatted ethanolic extract; IC_{50}, half maximal inhibitory concentration. Groups bearing the same letter have no significant difference between them. *Significantly different from different extract and standard value ($P<0.05$).

Discussion

This study demonstrates that the leaves of *H. pendula* are a rich source of phenolic compounds. The ethnological use of *H. pendula* externally and internally reflects its safety, which was confirmed by El-Gohary [9], wherein the LD_{50} of aqueous and alcoholic extracts from *H. pendula* seeds was 3.7 and 1.0 g/kg body weight, respectively. In a previous study, the methanolic extract of *H. pendula* leaves exhibited (95.5%) DPPH radical scavenging potential at 50 ppm [10]. Herein, DAEE and TEE showed an interesting and comparatively potent ABTS [half maximal inhibitory concentration (IC_{50}) 13.3 ±0.4 and 17.7±0.7 µg/ml] and NO (IC_{50}: 12.8±2.54 and 18.3±1.6 µg/ml) scavenging activity. Concurrently, the α-amylase inhibitory effect of both extracts was promising (IC_{50}: 6.1±0.554 and 14.4±0.681 µg/ml) as compared with acarbose and maltose (IC_{50}: 15.7±0.63 and 23.4± 0.975 µg/ml, respectively). DAEE was extremely the most effective α-amylase inhibitor of the tested compounds despite its lower phenolic and flavonoid content compared with TEE, indicating that the inhibitory effect is correlated to the structure of chemical constituents rather than their quantity. This prompted us to investigate the chemical composition of DAEE, which accounted for 25% w/w of dry plant, about eight flavonoids, six galloyl compounds along with two benzene acetic acid derivatives, which were isolated and identified for the first time from *H. pendula*, except for quercetin, kaempferol 3-*O*-gentiobioside, and harpulliaside A. Exclusively, the benzene acetic acid derivatives are mainly found in a genera of Sapindaceae subfamily Dodoneoideae reported for their selective anti-Gram (positive) and moderate NQO1 inhibitor activities [26,27].

According to the high-performance liquid chromatography analysis of defatted crude ethanol, MG was the predominant component in *H. pendula* leaves extracts followed by gallic acid (18 and 10 mg/g dry weight, respectively). The radical scavenging ability of MG was less than both extracts. Nevertheless, MG with IC_{50} values in $ABTS^{+}$ and NO assay (19.4±0.08 and 29.8± 1.00 µg/ml, respectively) showed higher scavenging effect than the standard vitamin C (IC_{50}: 21.2±0.24 and 39.5± 1.06 µg/ml, respectively). Its inhibitory capacity of α-amylase (with IC_{50}: 17.5±0.003 µg/ml) was much lower than that of maltose. The MG antioxidant activity has been reported in DPPH assay and $ABTS^{+}$ scavenging assay; the IC_{50} value was found to be 21.679 and 8.689 µg/ml, respectively, whereas, gallic acid IC_{50}

was 8.499 and 2.569 μg/ml, respectively [28]. In another DPPH assay it showed IC_{50} (2.25 μg/ml) [29], in addition, its immunomodulatory activity was demonstrated when a significant increase in leukocyte counts was observed in a mouse administered with MG at a concentration of 10 mg/kg body weight daily for 7 days [29]. Moreover, MG possessed antibacterial [30], anti-inflammatory [31], antitumor [32], and antiviral [33] *in vivo* activity. The reported *in vivo* LD_{50} for MG was 1700 mg/kg orally and in vitro IC_{50} 1.2 mm was [34]. Herein, MG scores additional NO scavenging capacity and an α-amylase suppression impact. The study represents MG as a lead for control of hyperglycemia and obesity with reasonable safety.Phenolics are widely distributed in food and plants reputed for their antioxidant activity [35,36]. The antioxidant activity of natural flavonoids is associated with the number and location of the hydroxyl group, the conjugation of 2,3-double bond with 4-oxo function in ring C, 3- and 5-hydroxy groups, 3,5,7-trihydroxy, *ortho*-catechol group (3′,4′-OH), and inversely affected by the glycosylation model (C-glycosides or O-glycosides) and position [35], which are present with our isolated flavonoids. Furthermore, the antioxidant activity of phenolic acid and gallic acid derivatives is related to the number and position of the hydroxyl group with respect to the carboxylic group (para-position maintains the scavenging activity). Moreover, the length of the ester chain of the substituted group inversely affects the scavenging activity due to the steric effects [37]. In contrast, some flavonoids, such as rutin, kaempferol and quercetin, have been previously reported to inhibit α-amylase. The glycosylation of flavonoids decreased the inhibitory effect against α-amylase, depending on the conjugation position and the class of sugar moiety, which may be due to the increasing molecular size and polarity, and transfer to the nonplanar structure [35]. Wang *et al*. [38] found that α-amylase inhibitory activity of quercetin was better than that of its glycosides. Another study reported that the monoglycosides of quercetin are stronger than their polyglycoside form (rutin) as α-amylase inhibitors [39]. Furthermore, Xiao *et al*. [40] reported that the affinities of gallic acid and its esters with α-amylase were determined as gallic acid>MG>ethyl gallate>propyl gallate due to hydrophobic forces.

NO is a molecule that our body produces from amino acid l-arginine by the enzymes in the vascular endothelial cells, phagocytes, and neuronal cells. NO is a free radical that plays many roles as an effector in various biological processes, including being a neuronal messenger, in vasodilatation, and in antitumor and antimicrobial activities. The NO toxicity increases greatly when it reacts with the superoxide radical, forming the highly reactive peroxynitrite anion ($ONOO^-$). Chronic exposure to the NO radical is associated with several inflammatory illnesses and carcinomas, including juvenile diabetes, arthritis, multiple sclerosis, and ulcerative colitis. NO inhibitors have been shown to have protective effects on inflammation and tissue damage associated with inflammatory diseases. NO has been shown to be directly scavenged by flavonoids [41,42].

Diabetes mellitus is one of the most important health problems with increasing incidence in rural populations worldwide characterized by chronic hyperglycemia. The consumption of vegetables is inversely associated with the risk of type 2 diabetes [43]. Natural plant sources have been inspected for their activity to deactivate glucose by various mechanisms either by decreasing the postprandial hyperglycemia, which is considered as one of the therapies oncoming for treating diabetes or by holding back the absorption of glucose through the inhibition of the carbohydrate hydrolyzing enzymes α-amylase and α-glucosidase in the digestive tract [44]. Inhibition of α-amylase seems to delay carbohydrate digestion, resulting in reduction in the rate of glucose absorption and consequently reducing the postprandial plasma glucose rise. Both α-amylase and α-glucosidase possess different inhibition kinetics, which seems to be due to structural differences related to the origin of the enzymes [45]. Indeed, the DAEE contains heterogeneous potent bioactive molecules that are responsible for antioxidant activity and α-amylase inhibitory effect.

Conclusion

H. pendula may be considered as an important source of biologically active secondary metabolites that can be developed into antidiabetic drugs and help in controlling hyperglycemia and weight gain. The preliminary results revealed the promising inhibitory effect of *H. pendula* DAEE, TEE extracts, and MG on carbohydrate metabolizing enzymes *in vitro* and may be considered as an exploratory marker for the potency of the plant extracts that enhance further *in-vivo* study.

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Conflicts of interest
There are no conflicts of interest.

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